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Targeted Pharmaceutical Nanocarriers for Cancer Therapy and Imaging

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Abstract

The use of various pharmaceutical nanocarriers has become one of the most important areas of nanomedicine. Ideally, such carriers should be specifically delivered (targeted) to the pathological area to provide the maximum therapeutic efficacy. Among the many potential targets for such nanocarriers, tumors have been most often investigated. This review attempts to summarize currently available information regarding targeted pharmaceutical nanocarriers for cancer therapy and imaging. Certain issues related to some popular pharmaceutical nanocarriers, such as liposomes and polymeric micelles, are addressed, as are different ways to target tumors via specific ligands and via the stimuli sensitivity of the carriers. The importance of intracellular targeting of drug- and DNA-loaded pharmaceutical nanocarriers is specifically discussed, including intracellular delivery with cell-penetrating peptides.

Keywords: Nanoparticles, nanocarriers, targeted delivery, cancer therapy, imaging

Introduction: Nanocarriers for Drug Delivery

Nanotechnology is expected to have a dramatic impact on medicine. The application of nanotechnology for treatment, diagnosis, monitoring, and control of biological systems is now often referred to as nanomedicine. Among many possible applications of nanotechnology in medicine, the use of various nanomaterials as pharmaceutical delivery systems for drugs, DNA, and imaging agents has gained increasing attention. Many varieties of nanoparticles are available,[1] such as different polymeric and metal nanoparticles, liposomes, niosomes, solid lipid particles, micelles, quantum dots, dendrimers, microcapsules, cells, cell ghosts, lipoproteins, and different nanoassemblies.

The paradigm of using nanoparticulate pharmaceutical carriers to enhance the in vivo efficiency of many drugs, anticancer drugs first of all, has well established itself over the past decade, in both pharmaceutical research and the clinical setting. Numerous nanoparticle-based drug delivery and drug targeting systems are currently developed or under development.[2,3] Their use aims to minimize drug degradation and inactivation upon administration, prevent undesirable side effects, and increase drug bioavailability and the fraction of drug delivered in the pathological area. In general, pharmaceutical drug carriers, especially those used for parenteral administration, are expected to be biodegradable, easy, and reasonably cheap to prepare; to have a small particle size; to possess a high loading capacity; to demonstrate prolonged circulation; and, ideally, to specifically or nonspecifically accumulate in required sites in the body.[4]

Some time ago, it was found that high-molecular-weight (40 kDa or higher), long-circulating macromolecules as well as various long-circulating nanoparticulate pharmaceutical carriers are capable of spontaneous accumulations in various pathological sites, such as solid tumors and infarcted areas, via the so-called enhanced permeability and retention (EPR) effect.[5,6] This effect is based on the fact that pathological vasculature, unlike vasculature of normal healthy tissues, is "leaky"—that is, penetrable for large molecules and even for small particles—which allows for their extravasation and accumulation in an interstitial tumor space. Such accumulation is additionally facilitated by the virtual lack of a lymphatic system, responsible for the drainage of macromolecules from normal tissues, in many tumors.[6] It has been found that the effective pore size in the endothelial lining of the blood vessels in most peripheral human tumors ranges from 200 nm to 600 nm in diameter, and the EPR effect allows for passive targeting to tumors based on the cutoff size of the leaky vasculature.[7]

We will illustrate here the large family of pharmaceutical nanocarriers with some examples. Among particulate drug carriers, liposomes, micelles, and polymeric nanoparticles are the most extensively studied and possess the most suitable characteristics for encapsulation of many drugs, genes, and diagnostic (imaging) agents. These drug carriers as well as any other pharmaceutical nanocarriers can be surface modified by a variety of different moieties to impart them with certain properties and functionalities. These functionalities are expected to provide nanocarriers: (1) prolonged

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circulation in the blood[8,9] and ability to accumulate in various pathological areas (eg, solid tumors) via the EPR effect (protective polymeric coating with polyethylene glycol [PEG] is frequently used for this purpose)[10,11]; (2) the ability to specifically recognize and bind target tissues or cells via the surface-attached specific ligand (monoclonal antibodies as well as their Fab fragments and some other moieties, eg, folate or transferrin, are used for this purpose)[12]; (3) the ability to respond to local stimuli characteristic of the pathological site by, for example, releasing an entrapped drug or specifically acting on cellular membranes under the abnormal pH or temperature in disease sites (this property could be provided by surface-attached pH- or temperature-sensitive components); and (4) the ability to penetrate inside cells bypassing lysosomal degradation for efficient targeting of intracellular drug targets (for this purpose, the surface of nanocarriers is additionally modified by cell-penetrating peptides). These are just the most evident examples. Some other specific properties can also be listed, such as an attachment of diagnostic moieties. Even the use of individual functionalities is already associated with highly positive clinical outcome; the success of Doxil, doxorubicin in a long-circulating PEG-coated liposome, is a good example.[13] Patient research showed the impressive effect of doxorubicin in PEG liposomes against metastatic breast carcinoma,[13-15] unresectable hepatocellular carcinoma,[16] cutaneous T-cell lymphoma,[17] sarcoma,[18] squamous cell cancer of the head and neck,[19] and ovarian cancer.[20] Liposomal lurtotecan was found to be effective in patients with topotecan-resistant ovarian cancer.[21]

Among the most popular and well-investigated drug carriers are liposomes (mainly, for the delivery of water-soluble drugs) and micelles (for the delivery of poorly soluble drugs) (Figure 1). Liposomes are artificial phospholipid vesicles that vary in size from 50 to 1000 nm and can be loaded with a variety of water-soluble drugs (into their inner aqueous compartment) and sometimes even with water-insoluble drugs (into the hydrophobic compartment of the phospholipid bilayer). For more than 2 decades they have been considered to be promising drug carriers.[22,23] They are biologically inert and completely biocompatible, and they cause practically no toxic or antigenic reactions; drugs included in liposomes are protected from the destructive action of external media. The use of targeted liposomes, that is, liposomes selectively accumulating inside an affected organ or tissue, increases the efficacy of the liposomal drug and decreases the loss of liposomes and their contents in the reticuloendothelial system (RES) (Table 1). To obtain targeted liposomes, many protocols have been developed to bind corresponding targeting moieties, including antibodies, to the liposome surface without affecting the liposome integrity and antibody properties.[22,24] However, the approach with immunoliposomes may nevertheless be limited because of their short life in the circulation.[25] Dramatically better accumulation can be achieved if the circulation time of liposomes is extended, increasing the total quantity of immunoliposomes passing through the target and increasing their interactions with target antigens. This is why long-circulated (usually, coated with PEG, ie, PEGylated) liposomes have attracted so much attention over the last decade.[8] It was demonstrated[26] that the unique properties of long-circulating and targeted liposomes could be combined in 1 preparation in which antibodies or other specific binding molecules had been attached to the water-exposed tips of PEG chains.[27]

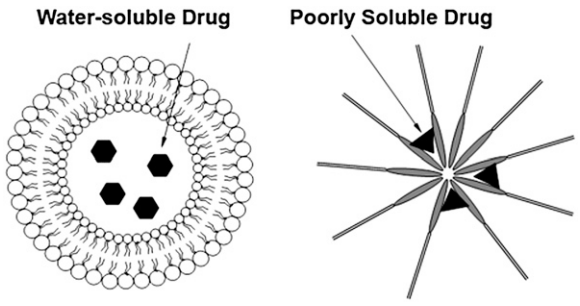

Figure 1. Schematic pictures of the liposome (left) and micelle (right) and their load with various drugs.

The development of drug nanocarriers for poorly soluble pharmaceuticals is an important task, particularly because large proportions of new drug candidates emerging from high-throughput drug screening initiatives are water-insoluble, but there are some unresolved issues. The therapeutic application of hydrophobic, poorly water-soluble agents is associated with some serious problems, since low water-solubility results in poor absorption and low bioavailability.[28] In addition, drug aggregation upon intravenous administration of poorly soluble drugs might lead to such complications as embolism[29] and local toxicity.[30] On the other hand, the hydrophobicity and low solubility in water appear to be intrinsic properties of many drugs,[31] since it helps a drug molecule to penetrate a cell membrane and reach important intracellular targets.[32,33] To overcome the poor solubility of certain drugs, the use of various micelle-forming surfactants in formulations of insoluble drugs is suggested. This is why micelles, including polymeric micelles,[34] are another promising type of pharmaceutical carrier. Micelles are colloidal dispersions with a particle size between 5 nm and 100 nm. An important property of micelles is their ability to increase the solubility and bioavailability of poorly soluble pharmaceuticals. The use of certain special amphiphilic molecules as micelle building blocks can also extend the blood half-life upon intravenous administration. Because of their small size (5-100 nm), micelles demonstrate spontaneous penetration into the interstitium in the body compartments with leaky vasculature (tumors and infarcts) by the EPR effect—a form of selective

Table 1. Some Examples of Liposomal Drugs Approved for Clinical Application or Undergoing Clinical Evaluation for Cancer Therapy

Active Drug (and product name for liposomal preparation)	Indications
Daunorubicin (DaunoXome)	Kaposi's sarcoma
Doxurubicin (Mycet)	Combinational therapy of recurrent breast cancer
Doxorubicin in polyethylene glycol liposomes (Doxil, Caelyx)	Refractory Kaposi's sarcoma; ovarian cancer; recurrent breast cancer
Vincristine (Onco TCS)	Non-Hodgkin's lymphoma
Lurtotecan (NX211)	Ovarian cancer
All-trans retinoic acid (Altragen)	Acute promyelocytic leukemia; non-Hodgkin's lymphoma; renal cell carcinoma; Kaposi's sarcoma
Platinum compounds (Platar)	Solid tumors
Annamycin	Doxorubicin-resistant tumors
DNA plasmid encoding HLA-B7 and β2 microglobulin (Allovectin-7)	Metastatic melanoma
Liposomes for various drugs and diagnostic agents (LipoMASC)	Various applications

delivery termed "passive targeting."[11] It has been repeatedly shown that micelle-incorporated anticancer drugs, such as adriamycin (see, eg, Kwon and Kataoka[35]) accumulate better in tumors than in nontarget tissues, thus minimizing undesired drug toxicity toward normal tissue. Diffusion and accumulation parameters for drug carriers in tumors have recently been shown to be strongly dependent on the cutoff size of the tumor blood vessel wall, and the cutoff size varies for different tumors.[7] Specific ligands (eg, antibodies and/or certain sugar moieties) can be attached to the water-exposed termini of hydrophilic blocks.[27] In the case of targeted micelles, local release of a free drug from micelles in the target organ should lead to increased efficacy of the drug, while the stability of the micelles en route to the target organ or tissue should contribute better drug solubility and toxicity reduction, because of less interaction with nontarget organs.

By virtue of their small size and by functionalizing their surface with synthetic polymers and appropriate ligands, nanoparticulate carriers can be targeted to specific cells and locations within the body after intravenous or subcutaneous injection. Such approaches may enhance detection sensitivity in medical imaging, improve therapeutic effectiveness, and decrease side effects. Some of the carriers can be engineered in such a way that they can be activated by changes in the environmental pH, by chemical stimuli, by the application of a rapidly oscillating magnetic field, or by application of an external heat source.[36-39] Such modifications offer control over particle integrity, drug delivery rates, and the location of drug release, for example, within specific organelles. Some carriers are being designed with a focus on multifunctionality, targeting cell receptors and delivering drugs and biological sensors simultaneously. Some include the incorporation of 1 or more nanosystems within other carriers, as in micellar encapsulation of quantum dots; this allows to follow their inherent nonspecific adsorption and aggregation in biological environments.[40]

Targeting Cancer

Role of Nanocarrier Longevity in the Blood

A serious limitation with all pharmaceutical nanocarriers is that the body normally treats them as foreign particles, so they become easily opsonized and removed from the circulation before completion of their function. Thus, one of the most important properties of any pharmaceutical nanocarrier loaded with any anticancer drug is its longevity, and long-circulating pharmaceuticals and pharmaceutical carriers are currently an important and still growing area of biomedical research (see, eg, Cohen and Bernstein,[3] Lasic and Martin,[8] Torchilin,[12] Trubetskoy and Torchilin,[41] and Torchilin[42]). There are quite a few important reasons for making long-circulating drugs and drug carriers. One of them is to maintain a required level of a pharmaceutical agent in the blood for extended time intervals. Then, long-circulating drug-containing microparticulates or large macromolecular aggregates can slowly accumulate (EPR effect, also termed "passive" targeting or accumulation via an impaired filtration mechanism, see above) in pathological sites with affected and leaky vasculature (eg, tumors, inflammations, infarcted areas), and facilitate drug delivery in those areas.[11,43,44] See the schematic of this phenomenon in Figure 2. In addition, the prolonged circulation can help to achieve a better targeting effect for targeted (specific ligand-modified) drugs and drug carriers, allowing more time for their interaction with the target[42] because of a larger number of passages of targeted pharmaceuticals through the target with the blood.

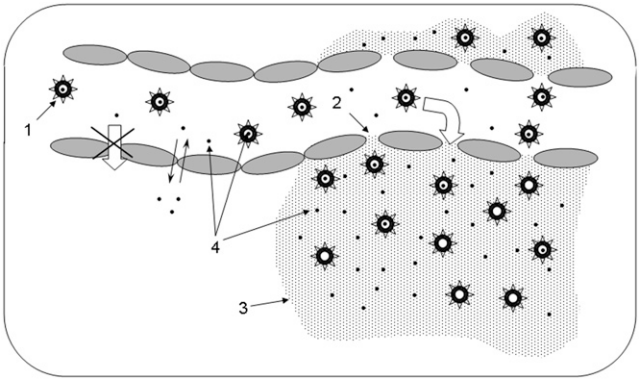

Figure 2. Enhanced permeability and retention (EPR) effect. Long-circulating drug carriers (1) penetrate through the leaky pathological vasculature (2) into the tumor interstitium (3) and degrade there, releasing a free drug (4) and creating its high local concentration.

Chemical modification of pharmaceutical nanocarriers with certain synthetic polymers, such as PEG, is the approach most frequently used to impart in vivo longevity to drug carriers, as was first suggested for liposomes.[45-49] Hydrophilic polymers have been shown to protect individual molecules and solid particulates from interaction with different solutes. The term "steric stabilization" has been introduced to describe the phenomenon of polymer-mediated protection.[50] On the biological level, coating nanoparticles with PEG sterically hinders interactions of blood components with their surface and reduces the binding of plasma proteins with PEGylated nanoparticles, as was demonstrated for liposomes.[47,51-55] This approach prevents drug carrier interaction with opsonins and slows down their capture by the RES.[25] The mechanisms by which PEG prevents opsonization include shielding of the surface charge, increased surface hydrophilicity,[56] enhanced repulsive interaction between polymer-coated nanocarriers and blood components,[57] and formation of the polymeric layer over the particle surface, which is impermeable for large molecules of opsonins even at relatively low polymer concentrations.[56,58] As a protecting polymer, PEG provides a very attractive combination of properties: excellent solubility in aqueous solutions; high flexibility of its polymer chain; very low toxicity, immunogenicity, and antigenicity; lack of accumulation in RES cells; and minimal influence on specific biological properties of modified pharmaceuticals.[59-62] It is also important that PEG is not biodegradable and subsequently does not form any toxic metabolites. PEG molecules with a molecular weight below 40 kDa are readily excretable from the body via the kidneys. PEG is also easily commercially available in a variety of molecular weights, although PEGs that are normally used for the modification of drug carriers have a molecular weight from 1000 to 20 000 Da. Currently, there are many chemical approaches to synthesizing activated derivatives of PEG and to coupling these derivatives with a variety of drugs and drug carriers (see reviews by Zalipsky,[59] Veronese,[63] and Torchilin[64]).

PEGylated polymeric nanoparticles can also be prepared based on the block-copolymer of PEG and a hydrophobic block, such as polylactide-glycolide (PLAGA).[4,65,66] Using PLAGA-PEG copolymer, one can prepare long-circulating particles with an insoluble (solid) PLAGA core and a water-soluble PEG shell covalently linked to the core.[4,66] Clearance and liver accumulation patterns reveal that the higher the content of PEG blocks, the slower the clearance and the better protection from liver uptake. Similar effects on longevity and biodistribution of microparticular drug carriers might be achieved by direct chemical attachment of protective polyethylene oxide chains onto the surface of preformed particles.[67] The surface of poly(lactic-glycolic acid) or PLAGA microspheres was also modified by adsorption of the polylysine-PEG copolymer, which resulted in a dramatic decrease of plasma protein adsorption on modified nanoparticles.[68] Similarly, coating polycyanoacrylate particles with PEG resulted in their increased longevity in the circulation, allowing for their diffusion into even the brain tissue.[69,70] Fluorouracil-containing dendrimer nanoparticles modified with PEG demonstrated better drug retention and less hemolytic activity.[71]

The most significant biological consequence of nanocarrier modification with protecting polymers is a sharp increase in circulation time and decrease in RES (liver) accumulation.[12,45,58] This fact is very important clinically, since various long-circulating nanocarriers have been shown to effectively accumulate in many tumors via the EPR effect.[11,43,44,72] Long-circulating liposomes were prepared containing various anticancer agents, such as doxorubicin, arabinofuranosylcytosine, adriamycin, and vincristine.[73-75] PEG-liposome-incorporated doxorubicin has already demonstrated very good clinical results.[44,76,77] From a pharmacokinetic point of view, the association of drugs with any nanocarrier has pronounced effects: delayed drug absorption, restricted drug biodistribution, decreased volume of drug biodistribution, delayed drug clearance, and retarded drug metabolism.[78,79] The presence of protective polymer on the carrier surface changes all these parameters still further.[12,47]

Tumor-Targeted Specific Ligands on Long-Circulating Nanocarriers

To achieve better selective targeting by PEG-coated liposomes or other particulates, targeting ligands were attached to nanocarriers via the PEG spacer arm, so that the ligand was extended outside of the dense PEG brush, excluding steric hindrances for its binding to the target receptors. With this in mind, potential ligands were attached to the activated far (distal) ends of some liposome-grafted polymeric

chains.[27,80] Since PEG-lipid conjugates used for the steric protection of liposomes and other pharmaceutical nanocarriers and for the preparation of polymeric micelles are derived from methoxy-PEG and carry only nonreactive methoxy terminal groups, several attempts have been made to functionalize PEG tips in PEG-lipid conjugates. For this purpose, several types of end-group functionalized lipopolymers of the general formula X-PEG-PE[59,81] were introduced, where X represents a reactive functional group-containing moiety, while PEG-PE represents the conjugate of polyethylene (PE) and PEG. Most of the end-group functionalized PEG lipids were synthesized from heterobifunctional PEG derivatives containing hydroxyl and carboxyl or amino groups. Typically, the hydroxyl end-group of PEG was derivatized to form a urethane attachment with the hydrophobic lipid anchor, PE, while the amino or carboxyl groups were used for the conjugation reaction or for further functionalization. To further simplify the coupling procedure and to make it applicable for single-step binding of a large variety of amino group–containing ligands (including antibodies, proteins, and small molecules) to the distal end of nanocarrier-attached polymeric chains, amphiphilic PEG derivative, p-nitrophenylcarbonyl-PEG-PE (pNP-PEG-PE), was introduced.[27,82,83] pNP-PEG-PE readily adsorbs on hydrophobic nanoparticles or incorporates into liposomes and micelles via its phospholipid residue, and it easily binds any amino group–containing compound via its water-exposed pNP group, forming a stable and nontoxic urethane (carbamate) bond. The reaction between the pNP group and the ligand amino group proceeds easily and quantitatively at pH around 8.0, while excessive free pNP groups are easily eliminated by spontaneous hydrolysis.

Although various monoclonal antibodies have been shown to deliver liposomes and other nanocarriers to many targets, attempts to optimize the properties of immunoliposomes and long-circulating immunoliposomes continue to be made. The majority of research relates to cancer targeting, which uses a variety of antibodies. Internalizing antibodies are required to achieve significantly improved therapeutic efficacy of antibody-targeted liposomal drugs, as was shown using B-lymphoma cells and internalizable epitopes (CD19) as an example.[84] An interesting concept was developed to target *HER2*-overexpressing tumors using anti-*HER2* liposomes.[85] In all studied *HER2*-overexpressing models, immunoliposomes showed potent anticancer activity superior to that of control nontargeted liposomes. In part, this superior activity was attributed to the ability of the immunoliposomes to deliver their load inside the target cells via receptor-mediated endocytosis, which is obviously important if the drug's site of action is located inside the cell. Antibody CC52 against rat colon adenocarcinoma CC531 attached to PEGylated liposomes provided specific accumulation of liposomes in a rat model of metastatic CC531.[86]

We obtained a nice illustration of how the addition of the targeting function onto a long-circulating drug-loaded nanocarrier can significantly enhance the activity of a drug when we demonstrated that the nucleosome-specific monoclonal antibody capable of recognizing various tumor cells via the tumor cell surface-bound nucleosomes improved Doxil targeting to tumor cells and increased its cytotoxicity[87] both in vitro and in vivo (Figure 3). *GD2*-targeted immunoliposomes with the novel antitumoral drug fenretinide, which induced apoptosis in neuroblastoma and melanoma cell lines, demonstrated strong antineuroblastoma activity both in vitro and in vivo in mice.[88] scFv antibody-modified liposomes were used to target cytotoxic drugs to biological targets, such as ED-B fibronectin.[89] The combination of immunoliposome and endosome-disruptive peptide improves cytosolic delivery of liposomal drug, increases cytotoxicity, and offers a new approach to constructing targeted liposomal systems, as shown in the case of diphtheria toxin A chain incorporated with the pH-dependent fusogenic peptide diINF-7 into liposomes specific toward ovarian carcinoma.[90]

Surface modification with antibodies was also applied to make some other pharmaceutical nanocarriers cancer-targeted (see Brannon-Peppas and Blachette[91] for review). Nanoparticles made of poly(lactic acid) were surface modified with PEG and with antitransferrin receptor monoclonal antibody to produce PEGylated immunoparticles with a size of ~120 nm that contained about 65 bound antibody molecules per single particle.[92] Mammalian cells (NIH3T3, 32D, Ba/F3, and hybridoma 9E10) were surface modified with distal terminus-activated oleyl-PEG, and various proteins

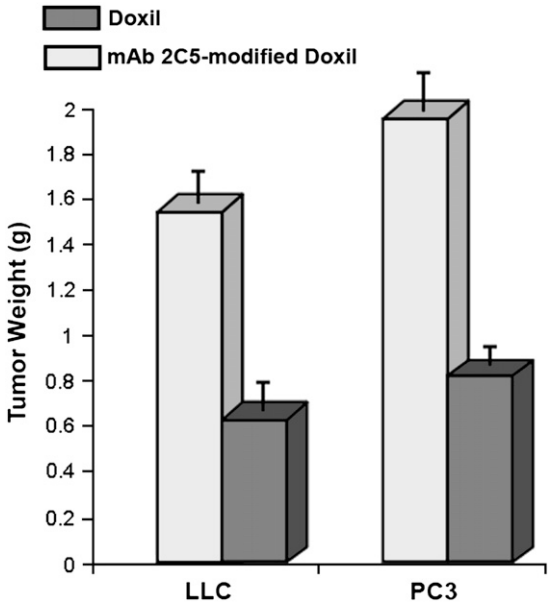

Figure 3. Antitumor activity in vivo in mice of tumor-specific antibody-modified Doxil compared with unmodified Doxil. LLC indicates Lewis lung carcinoma; PC3, line of prostate carcinoma.

(streptavidin, green fluorescent protein [EGFP], and antibody) were successfully attached to the activated PEG termini.[93] A similar combination of longevity and targetability can also be achieved by using some other specific ligands attached to long-circulating preparations. Thus, since transferrin (Tf) receptor (TfR) is overexpressed on the surface of many tumor cells, antibodies against TfR as well as Tf itself are among the ligands popular for targeting various nanoparticular drug carriers, including liposomes, to tumors and inside tumor cells.[94] Recent studies involve the coupling of Tf to PEG on PEGylated liposomes in order to combine longevity and targetability for drug delivery into solid tumors.[95] A similar approach was applied to deliver into tumors agents for photodynamic therapy, including hypericin,[96,97] and for intracellular delivery of cisplatin into gastric tumors.[98] Tf[99] as well as anti-TfR antibodies[100,101] were also used to facilitate gene delivery into cells by cationic liposomes. Tf-mediated liposome delivery was also successfully used for brain targeting. Immunoliposomes with OX26 monoclonal antibody to the rat TfR were found to concentrate on brain microvascular endothelium.[102]

Targeting tumors with folate-modified nanocarriers is another popular approach, since folate receptor (FR) expression is frequently overexpressed in many tumor cells.[103-106] Liposomal daunorubicin[107] and doxorubicin[108] were delivered into various tumor cells via folate receptor and demonstrated increased cytotoxicity. Folate-targeted liposomes have been suggested as delivery vehicles for boron neutron capture therapy[109] and are used also for targeting tumors with haptens for tumor immunotherapy.[110] Folate was also attached to the surface of cyanoacrylate-based nanoparticles via activated PEG blocks.[111] Similarly, PEG-polycaprolactone-based particles were surface-modified with folate and, after being loaded with paclitaxel, demonstrated increased cytotoxicity.[112] Superparamagnetic magnetite nanoparticles were modified with folate (with or without PEG spacer) and demonstrated better uptake by cancer cells, a finding useful for both diagnostic (magnetic resonance [MR] imaging agents) and therapeutic purposes.[113,114]

As with other delivery systems, the drug delivery potential of polymeric micelles—carriers for poorly soluble anticancer drugs—may also be still further enhanced by attaching targeting ligands to the micelle surface. The attachment of various specific ligands to the water-exposed termini of hydrophilic blocks could be used to improve the targeting of micelles and micelle-incorporated drugs and DNA.[115] Among those ligands are various sugar moieties,[116] transferrin,[117] and folate residues[118] since many target cells, especially cancer cells, overexpress appropriate receptors (eg, transferrin and folate receptors) on their surface. Thus, it was shown that galactose- and lactose-modified micelles made of PEG-polylactide copolymer specifically interact with lectins, thus modeling targeting delivery of the micelles to hepatic sites.[116,119] Transferrin-modified micelles based on PEG and poly(ethyleneimine) (PEI) sized between 70 and 100 nm are expected to target tumors with overexpressed transferrin receptors.[117] Mixed micelle-like complexes of PEGylated DNA and PEI modified with transferrin[120,121] were designed for enhanced DNA delivery into cells overexpressing the same transferrin receptors. A similar targeting approach was successfully tested with folate-modified micelles.[122] Poly(L-histidine)/PEG and poly(L-lactic acid)/PEG block copolymer micelles carrying folate residues on their surface were shown to be efficient for the delivery of adriamycin to tumor cells in vitro, demonstrating the potential for solid tumor treatment and combined targetability and pH sensitivity.[123]

The search for new ligands for cancer targeting concentrates around specific receptors overexpressed on cancer cells. Thus, liposome targeting to tumors has been achieved by using vitamin and growth factor receptors.[124] Vasoactive intestinal peptide (VIP) was used to target PEG liposomes with radionuclides to VIP receptors of the tumor, which enhanced breast cancer inhibition in rats.[125] PEG liposomes were targeted by arginine-glycine-aspartate peptides to integrins of tumor vasculature and, being loaded with doxorubicin, demonstrated increased efficiency against C26 colon carcinoma in a murine model. A similar angiogenic homing peptide was used for targeted delivery to vascular endothelium of drug-loaded liposomes in experimental treatment of tumors in mice.[126] Epidermal growth factor receptor (EGFR)–targeted immunoliposomes were delivered to a variety of tumor cells overexpressing EGFR.[127] Mitomycin C in long-circulating hyaluronan-targeted liposomes increases Mitomycin C activity against tumors overexpress hyaluronan receptors.[128] Studies also continue with galactosylated liposomes used to target drugs to the liver for therapy of liver tumors or metastases.[129] Cisplatin-loaded liposomes specifically binding chondroitin sulfate, which is overexpressed in many tumor cells, were used for successful suppression of tumor growth and metastases in vivo.[130] Mannosylated liposomes with muramyl dipeptide significantly inhibited liver metastases in tumor-bearing mice.[131]

Targeting via Stimuli Sensitivity

The development of stimuli-sensitive nanocarriers is a hot issue in nanomedicine for cancer. The concept is based on the fact that tumors normally have a lower pH value and a higher temperature than normal tissue, and stimuli-sensitive nanocarriers can be built releasing the incorporated drug only when subjected to these "special" conditions of the tumor. One has to realize that the stability of PEGylated nanocarriers may not always be favorable for drug delivery to and into tumor cells. In particular, if drug-containing

nanocarriers accumulate inside the tumor, they may be unable to release the drug easily to kill the tumor cells. Likewise, if the carrier has to be taken up by a cell via an endocytic pathway, the presence of the PEG coat on its surface may preclude the contents from escaping the endosome and being delivered into the cytoplasm. To solve these problems, for example, in the case of long-circulating liposomes, the chemistry was developed to detach PEG from the lipid anchor in the desired conditions. Labile linkage that would degrade only in the acidic conditions characteristic of the endocytic vacuole or the acidotic tumor mass can be based on the diortho esters,[132] vinyl esters,[133] cysteine-cleavable lipopolymers,[134] double esters, and hydrazones that are quite stable at pH around 7.5 but are hydrolyzed relatively fast at pH values of 6 and below.[132,135,136] Polymeric components with pH-sensitive (pH-cleavable) bonds are used to produce stimuli-responsive drug delivery systems that are stable in the circulation or in normal tissues but acquire the ability to degrade and release the entrapped drugs in body areas or cell compartments with lowered pH, such as tumors, or cell cytoplasm or endosomes.[137-139] A variety of liposomes[140,141] and polymeric micelles[123,142,143] have been described that include the components with acid-labile bonds. Serum-stable, long-circulating PEGylated pH-sensitive liposomes were also prepared using the combination of PEG and the pH-sensitive terminally alkylated copolymer of N-isopropylacrylamide and methacrylic[139] on the same liposome, since the attachment of the pH-sensitive polymer to the surface of liposomes might facilitate liposome destabilization and drug release in compartments with decreased pH values. The combination of pH sensitivity and specific ligand targeting for cytosolic drug delivery using decreased endosomal pH values was described for folate- and Tf-targeted liposomes.[144-146] Mixed micelle made of pH-sensitive components (polyhistidine and polylactic acid) loaded with adriamycin and targeted to tumors with folate residue provided better drug release under lowered pH values and demonstrated better killing of MCF-7 cells in vitro.[123] Such micelles can be prepared from different components[147] and loaded with different drugs,[148] and they have already demonstrated their utility in vivo.[148] They have also been shown to suppress even drug-resistant tumor cells effectively.[149] Similar data have been also obtained with temperature-sensitive micelles.[150]

The stimuli sensitivity of PEG coats can also allow for the preparation of multifunctional drug delivery systems with temporarily "hidden" functions, which under normal circumstances are "shielded" by the protective PEG coat but become exposed after PEG detaches. A nanoparticular drug delivery system can be prepared so that it accumulates in the required organ or tissue and then penetrates inside target cells, delivering its load (drug or DNA) there. The initial target (tumor, infarct) accumulation could be achieved by passive targeting via the EPR effect or by specific ligand (antibody)-mediated active targeting, whereas the subsequent intracellular delivery could be mediated by certain internalizable ligands (folate, transferrin) or by cell-penetrating peptides (CPPs, eg, TAT or polyArg). When in the blood, the cell-penetrating function should be temporarily inactivated (sterically shielded) to prevent nonspecific drug delivery into nontarget cells. However, when inside the target, the nanocarrier loses its protective coat, exposes the cell-penetrating function, and provides intracellular drug delivery (Figure 4).[151] Systems like this one require that multiple functions attached to the surface of the nanocarrier work in a certain orchestrated and coordinated way. For the above system the following requirements have to be met: (1) the life of the carrier in the circulation should be long enough to fit EPR effect or targeted delivery requirements (ie, the PEG coat mediating the longevity function or the specific ligand mediating the targeting function should not be lost by the nanocarrier when in the circulation), and (2) the internalization of the carrier within the target cells should proceed sufficiently fast so as not to allow for carrier degradation and drug loss in the interstitial space (ie, local stimuli-dependent removal of the protective function and the exposure of the temporarily hidden second function should proceed fast).

Targeting Tumors for Diagnostic Visualization

Whatever imaging modality (gamma-scintigraphy, MR imaging, or computed tomography) is used, medical diagnostic imaging requires that sufficient intensity of a corresponding

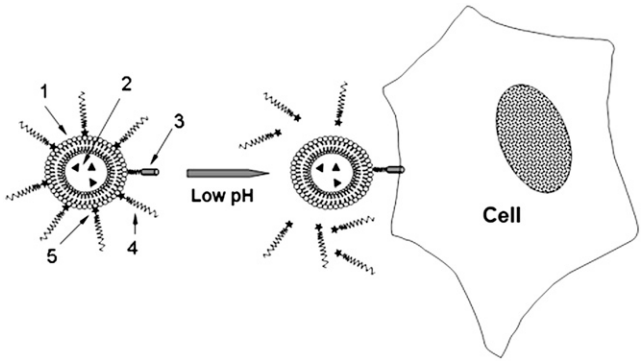

Figure 4. Targeting with the "hidden" function. The nanocarrier (1) is loaded with the drugs (2) and modified with the cell-penetrating function (3). Additionally, the nanocarrier is coated with a sterically protecting polymer (4) attached to the surface with the pH-sensitive bond (5). When in the blood, the cell-penetrating function is sterically shielded by the protective polymer chains, and the carrier accumulates in the tumor via the enhanced permeability and retention effect. Inside the tumor, local low pH causes the detachment of the protective polymer and the exposure of the cell-penetrating function, which then brings the carrier and the drug inside the tumor cells.

signal from an area of interest be achieved in order to differentiate this area from the surrounding tissues. Unfortunately, nonenhanced imaging techniques are useful only when relatively large tissue areas are involved in the pathological process. To solve the problem and to achieve a sufficient attenuation in the case of small lesions, contrast agents are used that are able to absorb certain types of signal (irradiation) much stronger than surrounding tissues. The contrast agents are specific for each imaging modality, and as a result of their accumulation in certain sites of interest, those sites may be easily visualized when the appropriate imaging modality is applied.[152] To further increase local spatial concentration of a contrast agent for better imaging, it was suggested that clinicians use certain nanoparticulate carriers able to carry multiple contrast moieties for efficient delivery of contrast agents to areas of interest and enhancement of signals from these areas.[153,154] Among nanocarriers for contrast agents, liposomes and micelles have received special attention because of their easily controlled properties and good pharmacological characteristics. Liposomes may incorporate contrast agents in both the internal aqueous compartment and the membrane. Two general approaches are used to prepare liposomes for gamma and MR imaging. First, the reporter metal is chelated into a soluble chelate (eg, diethylenetriamine pentaacetate [DTPA]) and then included in the interior of a liposome.[155] Alternatively, DTPA or a similar chelating compound may be chemically derivatized by the incorporation of a hydrophobic group, which can anchor the chelating moiety onto the liposome surface during or after liposome preparation.[156] Different chelators and different hydrophobic anchors were tried for the preparation of ^{111}In, ^{99m}Tc, Mn-, and Gd-liposomes.[157-164] In the case of MR imaging, for a better MR signal, all reporter atoms should be freely exposed for interaction with water. Membranotropic chelating agents—such as DTPA-stearylamine (DTPA-SA)[159] or DTPA-phosphatidyl ethanolamine (DTPA-PE)[156]—consist of the polar head containing a chelated paramagnetic atom, and the lipid moiety that anchors the metal-chelate complex in the liposome membrane. Liposomes with membrane-bound paramagnetic ion demonstrate also a reduced risk of leakage of potentially toxic metals in the body.

The amphiphilic chelating probes (paramagnetic Gd-DTPA-PE and radioactive ^{111}In-DTPA-SA) were also incorporated into PEG(5 kDa)-PE micelles and used in vivo for MR and scintigraphy imaging. In micelles, the lipid part of the molecule can be anchored in the micelle's hydrophobic core while a more hydrophilic chelate is localized on the hydrophilic shell of the micelle. The main feature that makes PEG-lipid micelles attractive for diagnostic imaging applications is their small size, which allows for good intratumoral accumulation of diagnostic micelles.

To further increase liposome load with diagnostic moieties, polychelating amphiphilic polymers (PAP) were synthesized consisting of the main chain with multiple side chelating groups capable of firm binding many reporter metal atoms and a hydrophobic terminal group, allowing for polymer adsorption onto hydrophobic nanoparticles or incorporation into hydrophobic domains of liposomes or micelles (Figure 5).[165] Such surface modification of nanocarriers allows for a sharp increase in the number of bound reporter metal atoms per particle and the image signal intensity. In the case of MR, metal atoms chelated into polymer side groups are directly exposed to the water environment, which enhances the relaxivity of the paramagnetic ions and leads to a corresponding enhancement of the vesicle contrast properties.[161,166,167]

An interesting example of the application of PAP-nanoparticles in vivo is the MR imaging of lymphatic system components with Gd-loaded nanocarriers (important for discovering metastases in lymph nodes). Liposomes and micelles have been studied as delivery vehicles to the lymphatic system.[168,169] It has been shown that radioactively labeled small negatively charged liposomes are the most efficient in targeting rat regional lymph nodes after subcutaneous administration.[170] The optimal diameter of liposomes that localize in the lymph nodes after peritoneal administration in rats is ~200 nm.[171] Liposomes loaded with chelated paramagnetic ions (mostly Gd, Dy, Mn, Fe) have been demonstrated to be useful as MR imaging contrast agents, mostly for the visualization of macrophage-rich tissues, such as the organs of the RES.[159,172]

In experimental rabbits, transverse scans obtained after subcutaneous administration of a suspension of Gd-PAP-liposomes into the right forepaw demonstrated that axillary and subscapular lymph nodes can be seen on a scan taken only 5 minutes postinjection.[166] The approach was additionally

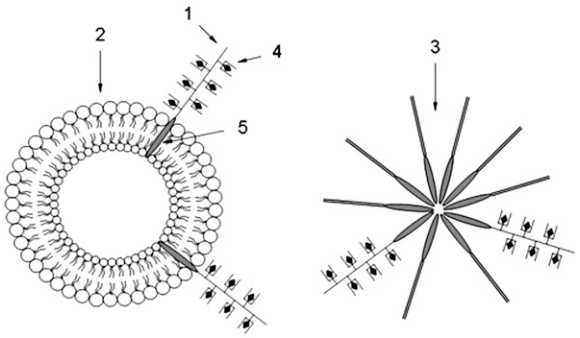

Figure 5. The incorporation of the amphiphilic polychelating polymer (1) into the liposome membrane (2) or micelle core (3). Each polychelating chain contains multiple chelating groups (4), which carry diagnostic heavy-metal atoms (eg, ^{111}In or ^{99m}Tc for gamma-scintigraphy or Gd or Mn for magnetic resonance imaging) and a hydrophobic tail (5) to anchor the liposome or micelle.

proved by fast and informative diagnostic visualization of VX_2 human sarcoma in rabbit popliteal lymph node, when with 200 nm of Gd-PAP-liposomes a tumor was clearly seen 10 minutes postinjection. The overall performance of Gd-PAP-liposomes or -micelles in lymph node visualization might be further improved by additional incorporation of amphiphilic PEG onto the liposome membrane or micelle surface, which can be explained by increased $1/T_1$ values of PEG-Gd-liposomes due to the presence of an increased amount of PEG-associated water protons in the close vicinity of chelated Gd ions located on the liposomal membrane.[173,174] In addition to the enhanced relaxivity, the coating of the liposome surface with PEG polymer can help in avoiding contrast agent uptake in the site of injection by resident phagocytic cells.

The ability of various PEG-liposomal formulations labeled with ^{67}Ga, ^{111}In, or ^{99m}Tc to localize in tumors has been demonstrated in a series of preclinical studies.[175] In fact, numerous successful tumor detection studies were done with contrast-loaded long-circulating liposomes using human tumor xenografts in nude mice.[176,177] Moreover, the clinical data on ^{111}In-labeled long-circulating liposomes are already available on the visualization of lung cancer,[178,179] head and neck cancers,[178,179] AIDS-related Kaposi's sarcoma,[180] skin cancer,[19] glioblastomas and metastatic brain tumors,[181] soft tissue sarcomas,[182] and other malignancies.[179]

^{111}In-DTPA-labeled PEGylated liposomes (prepared using the ^{111}In-oxine method) have also been used to study the biodistribution and the pharmacokinetics of long-circulating liposomes and to assess their applicability for the radioimaging of tumor localization and evaluation of different therapeutic treatment strategies against various types of cancers.[183,184] Clinically, successful tumor imaging was achieved in 15 of 17 patients with cancer (4 of 5 breast, 5 of 5 head and neck, 3 of 4 bronchus, 2 of 2 glioma, and 1 of 1 cervix).[179]

Koukourakis et al investigated the accumulation of ^{99m}Tc-DTPA-radiolabeled long-circulating liposomal doxorubicin (Doxil) in 10 patients with metastatic brain tumors and five patients with brain glioblastoma undergoing radiotherapy.[181] Radiolabeled Doxil accumulation was 13 to 19 times higher in the glioblastomas and 7 to 13 times higher in the metastatic lesions, as compared with the normal brain.

Belhaj-Tayeb et al have recently suggested an original method to encapsulate ^{99m}Tc-MIBI in preformed PEG liposomes.[185] They used an exchange with an efflux of K+ through the valinomycin ionophores, that is, an active encapsulation, which resulted in 50% encapsulation efficiency of ^{99m}Tc-MIBI in PEG liposomes. One hour postinjection in rats, PEG liposomes showed a 10 times higher activity in blood than free ^{99m}Tc-MIBI, whereas the activity of free ^{99m}Tc-MIBI in kidneys and bladder was markedly higher than that of encapsulated ^{99m}Tc-MIBI, indicating faster clearance of the free radiotracer. In the breast cancer (MCF7-ras)-bearing nude mice, PEG liposome uptake in tumors was 2 times that for the free ^{99m}Tc-MIBI. Overall, the ^{99m}Tc-MIBI-PEG liposomes demonstrated not only longer blood circulation time but also improved tumor-to-background ratio in the in vivo imaging.

Based on the fact that vasoactive intestinal peptide receptors (VIP-R) are approximately 5 times more expressed in human breast cancer compared with normal breast tissue, Dagar et al[125] used VIP, a 28-amino-acid mammalian neuropeptide, as a breast cancer targeting moiety for targeted imaging of breast cancer. VIP was covalently attached to the surface of long-circulating liposomes that encapsulated the ^{99m}Tc-HMPAO complex. It was found that the presence of VIP did not affect the size and ^{99m}Tc-HMPAO encapsulation ability of the liposomes and did not alter the pharmacokinetic profile of the PEGylated liposomal formulation. Liposomes with and without VIP on their surface accumulated at significantly higher quantities in breast cancer when compared with normal breast in rats, indicating the EPR-dependent passive targeting of these formulations to cancer tissues. Still, in breast cancer, ^{99m}Tc-HMPAO liposomes modified with VIP showed significantly more accumulation than did analogs without VIP. The tumor-to-nontumor ratio was also significantly higher for ^{99m}Tc-HMPAO liposomes modified with VIP than for VIP-free ^{99m}Tc-HMPAO liposomes, suggesting active targeting of VIP liposomes to breast cancer.

In general, nanoparticles are believed to be used as modular platforms to build a broad variety of targeted imaging agents, since multiple molecules of imaging agents and cancer-specific ligands can be assembled into a single nanoparticle.[153] Multifunctional magnetic nanocrystals coupled with a cancer-specific antibody, Herceptin, allowed for successful in vivo MR detection of cancer.[186] Anticancer antibodies were shown to effectively accumulate contrast agent–loaded pharmaceutical nanocarriers in tumors and provide for faster and better visualization (Figure 6).[187-189] Simultaneous cancer cell imaging and photodynamic therapy in the near-infrared region can be performed using gold nanorods[190] because of strong adsorption and scattering of electromagnetic radiation by noble-metal nanoparticles. The conjugation of cancer-specific antibody with such nanorods results in the preparation providing selective killing of cancer cells upon the exposure to a red laser at 800 nm. Use of the same principle was suggested for early optical detection of cancer with gold nanoshells.[191]

Miscellaneous Tumor Targeting

Photodynamic therapy (PDT) is a fast-developing modality for the treatment of superficial tumors, where photosensitizing

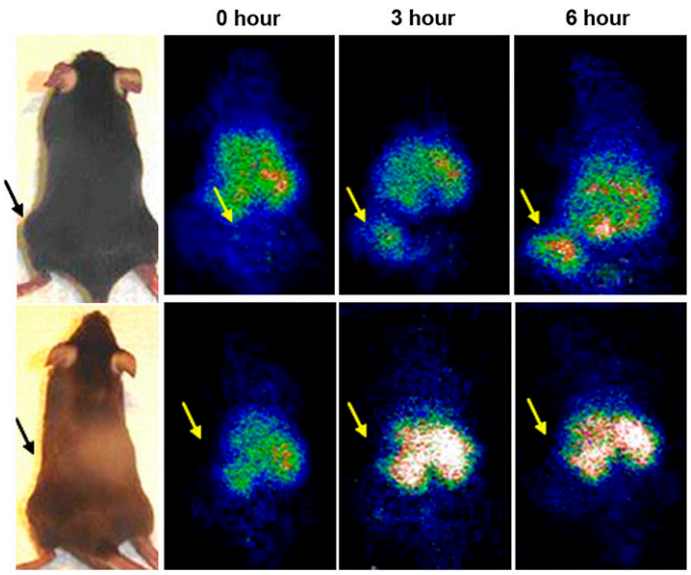

Figure 6. Whole body imaging of Lewis lung carcinoma tumor-bearing mice at different time points after the injection of ^{111}In-labeled polychelating amphiphilic polymers-containing polyethylene glycol liposomes. Upper row: 2C5-modified liposomes; bottom row: control unmodified liposome. Arrows indicate tumor locations. Notice the much faster accumulation of antibody-targeted liposomes in the tumor.

agents are used for photochemical eradication of malignant cells. In PDT, liposomes are used both as drug carriers and as enhancers (for a review, see Derycke and de Witte[192]). Targeting as well as the controlled release of photosensitizing agent in tumors may still further increase the outcome of the liposome-mediated PDT. Benzoporphyrin derivative encapsulated in polycation liposomes modified with cetyl-PEI was used for antiangiogenic PDT. This drug in such liposomes was better internalized by human umbilical vein endothelial cells and was found in the intranuclear region and associated with mitochondria.[193] Another porphyrin derivative (SIM01) in dimyristoyl phosphatidylcholine liposomes also gives better results in PDT, mainly because of better accumulation in the tumor (human adenocarcinoma in nude mice).[194] Targeting of a poorly soluble PDT agent, meso tetraphenylporphine, solubilized by polymeric micelles modified with a cancer-specific antibody resulted in more efficient killing of cancer cells under the conditions of PDT.[189]

Liposomes are also used for targeting of antisense oligonucleotides, in particular for neuroblastoma treatment, exemplified by coated cationic liposomes made of a central core of a cationic phospholipid bound to oligonucleotide, and an outer shell of neutral lipid. Such liposomes are additionally modified with a monoclonal antibody against neuroectoderma antigen and target antigen-positive cells both in vitro and in vivo.[195]

An interesting approach to the use of targeted liposomes loaded with enzymes in cancer therapy is their application for antibody-directed enzyme prodrug therapy based on the on-site activation of chemically modified inactive phospholipid derivatives of various anticancer and antiviral agents. The application of phospholipid prodrugs incorporated into liposome membranes brings several benefits[196]: the efficiency of the prodrug incorporation is high; prodrugs do not leak from the liposome into the aqueous phase; drugs are protected against metabolic degradation; and long-lasting therapeutic drug levels can be achieved. For specific generation of active cytotoxic molecules from inactive prodrugs in the vicinity of tumor cells, a conjugate of a tumor-specific antibody with an enzyme responsible for the conversion of a prodrug into the active drug is targeted toward the tumor. To increase the efficiency of the required enzyme in the tumor, rather than just "straight" antibody-enzyme conjugates, immunoliposomes were loaded with the required enzyme (immunoenzymosomes).[197]

An emerging area in cancer nanomedicine is now associated with the use of magnetic nanoparticles for different purposes. Thus, the suspensions of coated superparamagnetic nanoparticles were shown to be taken up by cancer cells and can kill those cancer cells after being subjected to an alternating current (AC) magnetic field because of developing hyperthermia.[198] The procedure is termed "magnetic thermal ablation" and has proved itself in various models, including breast cancer models.[199] A similar approach reduced tumor growth in the orthotopic model of prostate cancer in rats.[200] Moreover, the first clinical results from the use of magnetic nanoparticles for AC-mediated hyperthermia are quite encouraging.[201] This treatment can also be successfully combined with external radiation therapy, as was shown in the rat model.[202] It is especially important that this technique is applicable even to brain tumors, as was shown in rats with malignant glioma.[198] To further enhance the efficacy of this therapy, magnetic particles have been coupled with antitumor antibodies to provide better localization in the tumor, as was shown in an experiment with magnetic nanoparticles targeted to tumors, then subjected to hyperthermia with an external alternating magnetic field.[203]

Intracellular Delivery of Drugs and DNA for Cancer Therapy

Intracellular transport of biologically active molecules with therapeutic properties is one of the key problems in drug delivery. Many pharmaceutical agents need to be delivered intracellularly to exert their therapeutic action inside cytoplasm or onto individual cell organelles. Thus, intracellular drug delivery can overcome certain important limitations for drug action, such as multidrug resistance in cancer chemotherapy.

However, the very nature of cell membranes prevents proteins, peptides, and nanoparticulate drug carriers from entering cells unless there is an active transport mechanism, which is usually the case for very short peptides.[204] So far, multiple and only partially successful attempts have been made to bring various low-molecular-weight and macromolecular drugs and drug-loaded pharmaceutical carriers directly into the cell cytoplasm, bypassing the endocytic pathway, to protect drugs and DNA from lysosomal degradation, thus enhancing drug efficiency or DNA incorporation into the cell genome. However, even being safely delivered into the cell cytoplasm, drugs still have to find their way to specific organelles (nuclei, mitochondria), where they are expected to use their therapeutic potential. Various vector molecules promote the delivery of associated drugs and drug carriers inside the cells via receptor-mediated endocytosis.[85] This process involves attachment of the vector molecule and an associated drug carrier to specific ligands on target cell membranes, followed by the energy-dependent formation of endosomes. The problem, however, is that any molecule/particle entering the cell via the endocytic pathway and becoming trapped in the endosome eventually ends in the lysosome, where active degradation processes take place under the action of lysosomal enzymes. As a result, only a small fraction of unaffected substance appears in the cell cytoplasm. Thus, even if efficient cellular uptake via endocytosis is observed, the delivery of intact peptides and proteins is compromised by insufficient endosomal escape and subsequent lysosomal degradation.

pH-Sensitive Carriers

Quite a few approaches for cytosolic drug delivery with such pharmaceutical nanocarriers as liposomes and micelles have been developed. Among the different methods of liposomal content delivery into the cytoplasm[205] it was proposed that the liposome is made of pH-sensitive components and, after being endocytosed in the intact form, it fuses with the endovacuolar membrane under the action of lowered pH inside the endosome (below 6) and destabilizes the endosome, releasing its content directly into the cytoplasm.[206] Thus, endosomes become the gates from the outside into the cell cytoplasm.[207] Cellular drug delivery mediated by pH-sensitive liposomes is not a simple intracellular leakage from the lipid vesicle since the drug has to cross the endosomal membrane as well.[208] It is usually assumed that inside the endosome, the low pH and some other factors destabilize the liposomal membrane, which, in turn, interacts with the endosomal membrane, provoking its secondary destabilization and drug release into the cytoplasm. The presence of fusogenic lipids in the liposome composition, such as unsaturated dioleoylphosphatidylethanolamine (DOPE), with their ability to easily adopt an inverted hexagonal phase, is usually required to make liposomes pH-sensitive.[209] Importantly (since many current liposomal dosage forms are based on the use of long-circulating, PEGylated liposomes), long-circulating PEGylated DOPE-containing liposomes, although demonstrating decreased pH sensitivity compared with non-PEGylated liposomes, still effectively delivered their contents into the cytoplasm.[210]

The combination of liposome pH sensitivity and specific ligand targeting for cytosolic drug delivery using decreased endosomal pH values was described for both folate and Tf-targeted liposomes.[211] Additional modification of pH-sensitive liposomes with an antibody results in pH-sensitive immunoliposomes. The advantages of antibody-bearing pH-sensitive liposome include cytoplasmic delivery, targetability, and facilitated uptake (ie, improved intracellular availability) via receptor-mediated endocytosis. Successful application of pH-sensitive immunoliposomes has been demonstrated in delivery of a variety of molecules, including antitumor drugs.[212]

In addition to membrane-destabilizing lipid components, there exist a large number of membrane-destabilizing anionic polymers that also can enhance the endosomal escape of various drugs and biomacromolecules.[213] This family includes various carboxylated polymers, copolymers of acrylic and methacrylic acids, copolymers of maleic acid, and polymers and copolymers of N-isopropylacrylamide (NIPAM). Copolymers of NIPAM demonstrate a lower critical solution (solubility/insolubility switch) at physiological temperatures and when precipitated, destabilize biomembranes with which they interact.[214] Such polymers can be attached to the surface of drug-/DNA-loaded liposomes or polymeric micelles, allowing for endosomal destabilization and cytoplasmic escape.

Since micelle-based preparations of various poorly water-soluble drugs are considered to be promising dosage forms for such drugs, and since many of those drugs (eg, paclitaxel) target intracellular organelles, various micelles (polymeric micelles) have been prepared that can also demonstrate pH sensitivity and the ability to escape from endosomes. Thus, micelles prepared from PEG-poly(aspartate hydrazone adriamycin) easily release an active drug at lower pH values typical for endosomes and facilitate its cytoplasmic delivery and toxicity against cancer cells.[215] It is also possible to enhance the intracellular delivery of drug-loaded micelles by adding to their composition the lipid components used in membrane-destabilizing Lipofectin. Thus, PEG-lipid micelles, for example, carry a net negative charge,[10] which might hinder their internalization by cells. On the other hand, it is known that a net positive charge usually enhances the uptake of various nanoparticles by cells, and after endocytosis, the drug-/DNA-loaded particles could escape from the endosomes and enter a cell's cytoplasm through disruptive

interaction of the cationic lipid with endosomal membranes.[216] The compensation for the micelle negative charge by the addition of positively charged lipids to PEG-PE micelles could improve the uptake by cancer cells of drug-loaded mixed PEG-PE/positively charged lipid micelles. It is also possible that after the enhanced endocytosis, such micelles could escape from the endosomes and enter the cytoplasm of cancer cells. With this in mind, an attempt was made to increase the intracellular delivery and thus the anticancer activity of the micellar paclitaxel by preparing paclitaxel-containing micelles from the mixture of PEG-PE and Lipofectin lipids (LL).[217] When the cellular uptake of various fluorescently labeled micelles in adherent BT-20 cells was studied, it was found that while both "plain" PEG-PE micelles and PEG-PE/LL micelles were endocytosed by BT-20 cells, as confirmed by the presence of fluorescent endosomes in cells after 2 hours of co-incubation with fluorescently labeled micelles, in case of PEG-PE/LL micelles, endosomes became partially disrupted and their content was released into the cell cytosol. The addition of LL, facilitating the intracellular uptake and cytoplasmic release of paclitaxel-containing PEG-PE/LL micelles, resulted in a substantially increased level of cell death compared with that under the action of free paclitaxel or paclitaxel delivered using noncationic LL-free PEG-PE micelles. In BT-20 cancer cells, the IC50 values of free paclitaxel, paclitaxel in PEG-PE micelles, and paclitaxel in PEG-PE/LL micelles were 24.3, 9.5, and 6.4 μM, respectively. In A2780 cancer cells, the IC50 values for the same preparations were 22.5, 5.8, and 1.2 μM, respectively.

Delivery by CPPs

A novel and interesting approach to delivering various drug and DNA molecules and even drug-loaded nanoparticles inside cells for cancer therapy involves their modification with CPPs—proteins and peptides that can facilitate uptake through the cellular membranes—thereby enhancing the delivery of CPP-modified molecules inside the cell. During the last decade, several proteins and peptides have been found to traverse through the cellular membranes, delivering their cargo molecules into the cytoplasm and/or nucleus. Thus, 86-mer transactivating transcriptional activator (TAT) from HIV-1 was efficiently taken up by various cells, when added to the surrounding media.[218,219] Subsequently, this property of translocation was found in Antennapedia (Antp), a transcription factor of *Drosophila*,[220] and VP22, a herpes virus protein.[221] Their ability to translocate across the plasma membranes is confined to short sequences within these proteins of fewer than 20 amino acids, which are highly rich in basic residues. These peptides have been used for intracellular delivery of various cargoes with molecular weights several times greater than their own.[222] Cellular delivery using CPPs has several advantages over conventional techniques because it is efficient for a range of cell types and has a potential therapeutic application.[223]

Collectively, the recent data assume more than 1 mechanism for CPP-mediated intracellular delivery of various molecules and particles.[224] CPP-mediated intracellular delivery of large molecules and nanoparticles proceeds via the energy-dependent macropinocytosis, with subsequent enhanced escape from endosome into the cell cytoplasm,[224,225] while individual CPPs or CPP-conjugated small molecules penetrate cells via electrostatic interactions and hydrogen bonding and do not seem to depend on energy.[226] Direct contact between the translocating moiety and cell membrane or cell membrane–interacting proteoglycans is required for successful intracellular delivery.

Since traversal through cellular membranes represents a major barrier for efficient delivery of macromolecules inside cells, CPPs may ferry various molecules into mammalian cells in vitro and in vivo. The use of peptides and protein domains with amphipathic sequences for drug and gene delivery across cellular membranes is getting increasing attention. Covalent hitching of proteins, drugs, DNA, or other macromolecules onto CPPs may circumvent conventional limitations by allowing for transport of these compounds into a wide variety of cells in vitro and in vivo.[227-231]

Herpes simplex virus VP22 protein was used to deliver E2 protein into target cells. Overexpression of the E2 protein in cervical cancer cells can induce growth arrest and/or apoptotic cell death; thus, E2 might be useful in the treatment of cervical cancer. VP22-E2 fusion proteins induced apoptosis in transiently transfected human papillomavirus (HPV)–transformed cervical carcinoma cell lines. When COS-7 cells producing VP22-E2 were seeded into cultures of HPV-transformed cells, VP22-E2 entered the nonproducing cells and induced apoptosis. This suggests that the local delivery of VP22-E2 fusion proteins could be used to treat cervical cancer and other HPV-associated diseases.[232]

VP22 enhanced intercellular trafficking of thymidine kinase (TK) and amplified the TK/ganciclovir (GCV) killing effect, especially in the lower range of GCV concentrations, offering a new strategy to enhance the effectiveness of suicide gene therapy for the treatment of cancers.[233] Chimeric polypeptides of VP22 linked to the entire p53 protein were shown to spread between cells and accumulate in recipient cell nuclei. Also, the VP22-p53 chimeric protein efficiently induced apoptosis in p53 negative human osteosarcoma cells, resulting in a widespread cytotoxic effect.[234]

VP22 was also used to deliver oligonucleotides in vitro and in vivo. Complexes of VP22 with fluorescein-labeled oligonucleotides, termed "vectosomes," were efficiently taken up by cells and remained stable in the cell cytoplasm without any particular activity. These vectosomes were disrupted

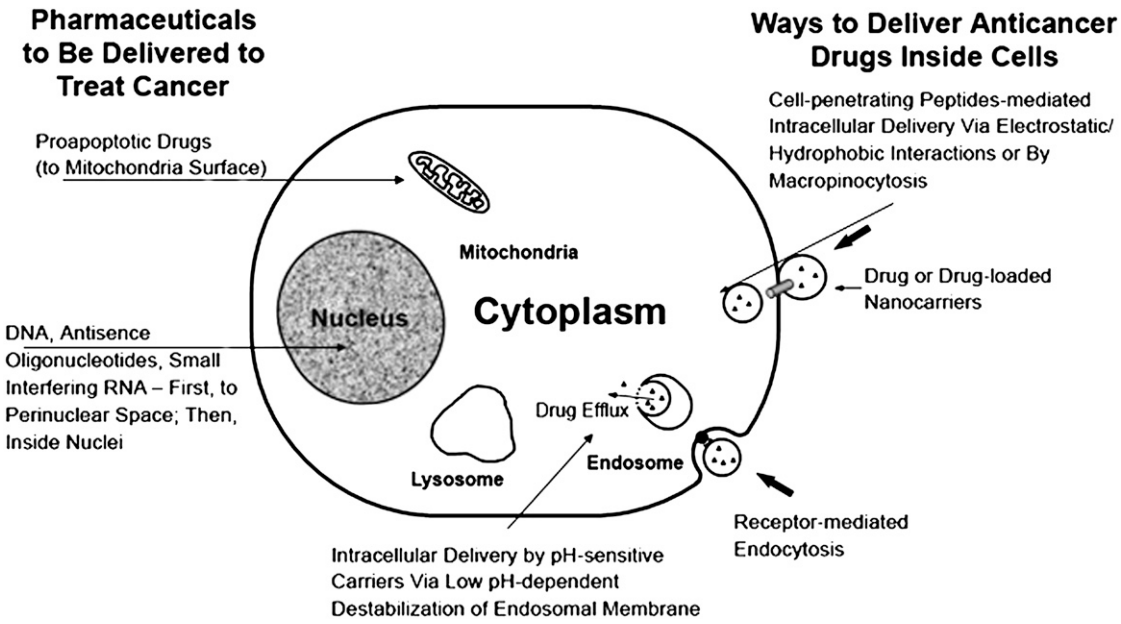

Figure 7. Schematics of intracellular drug delivery.

by light to release the antisense activity. Anti-c-Raf1 vectosomes were efficiently activated by light in vivo after injection into subcutaneous tumors implanted in nude mice and slowed down the tumor growth because of the strong inhibition of c-Raf1 protein expression, the antitumor activity being much higher than that of the antisense alone or of the different control vectosomes.[235]

Suicide gene therapy is a widely exploited approach for gene therapy of cancer and other hyperproliferative disorders. However, it is hampered by the relative inefficiency of TK gene transfer and its limited bystander effect. Fusion of TK to TAT protein transduction domain (PTD) imparted cell membrane translocating ability to the enzyme and significantly increased its cytotoxic efficacy. The enzyme was present extracellularly in the cells expressing TAT11-TK, associated with the cell surface heparan sulfate proteoglycans, and was released into the cell culture medium. The protein was then internalized by neighboring nonexpressing cells, which underwent apoptosis when treated with the nucleoside analog acyclovir. Thus, development of this approach was an important step in the establishment of TK suicide gene therapy.[236]

The attachment of TATp to a water-soluble synthetic macromolecule, N-(2-hydroxypropyl)methacrylamide copolymer, resulted in the cytoplasmic and nuclear delivery of the conjugate via nonendocytotic and concentration-independent processes, as opposed to conjugates without TATp, which accumulated in only endocytotic vesicles. Furthermore, the TATp-polymer-bound anticancer drug doxorubicin was delivered inside the cytoplasm, providing the possibility for the development of polymer-based systems for the cytoplasmic delivery of therapeutic molecules.[237,238]

Another growing application of transduction technology is in the field of cancer therapy, where the transduction methodology appears to circumvent the problems encountered with the conventional chemotherapeutic regimens, such as nonspecificity and exclusion of drugs by efflux transporters in multidrug-resistant cells. The transduction domains of Antennapedia and TAT have been linked to the tumor suppressor peptide p53, which enhanced the accumulation of p53 in the tumor cells and activated the apoptotic genes for the selective killing of tumor cells both in vitro and in vivo.[239-241] The TAT peptide has also been used to deliver proteins that modulate the cell cycle and arrest tumor growth.[242,243] Another approach to selectively killing tumor cells is transducing dendritic cells with tumor antigens to generate cytotoxic lymphocytes that eradicate tumors. The TAT transduction domain was used to transduce dendritic cells with ovalbumin, a recombinant model tumor-associated antigen. The transduced dendritic cells generated cytotoxic lymphocytes against tumors.[244] Immunization with transduced dendritic cells imparted an antitumor immunity and inhibited lung metastases in a 3-day tumor model.[245] Some approaches currently used for intracellular delivery are shown in Figure 7.

Conclusion

Even a rather brief review of what is going on in the field of tumor-targeted pharmaceutical nanocarriers shows the breadth of this approach. Significant information has accumulated regarding the most convenient carrier systems (eg, liposomes) and possible ways of using them for the targeted delivery of drugs, imaging agent, and genes into tumors.

The most important problem is now associated with the translation of various successfully proven experimental concepts into clinical practice. With evident achievements in the clinical use of some first-generation anticancer nanomedicines (Doxil being a good example), one can expect the appearance of "real" targeted anticancer nanomedicines in the not-too-distant future.

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